IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Atty. Docket: BARENHOLZ14

In re Application of:	)	Conf. No.: 4072
	)	
Yechezkel BARENHOLZ et al.	)	Art Unit: 1648
	)	
Appln. No.: 10/560,932	)	Examiner: J. L. Epps-Smith
	)	
Filed: May 5, 2006	)	Washington, D.C.
	)	
For: SPHINGOLIPIDS POLYALKYLAMINE CONJUGATES…)		October 12, 2011

DECLARATION UNDER 37 CFR 1.132

I, the undersigned Kirill Makedonski hereby declare and state as follows:

I have a PhD in biochemistry from Hebrew University, Jerusalem, Israel. I am Research Associate and Project Manager in the Laboratory of Membrane and Liposome research, Hadassah Medical Shool, Hebrew University, Jerusalem, Israel, which laboratory is headed by Prof. Yechezket Barenholz. A true and correct copy of my curriculum vitae is attached hereto as appendix A.

I have been asked to explain the experimentation that has taken place in our laboratory that proves the superiority of sphingoid-polyalkylamine conjugates with two polyalkylamine chains over sphingoid-polyalkylamine conjugates with only a single polyalkylamine chain.

N-palmitoyl-D-erythro sphingosyl-1-3-spermine (PCDCS) (Figure 1) is a cationic lipid that was designed in our Lab and produced by Biolab Ltd. This molecule has two polyalkylamine chains with a total of two primary amines that are positively charged in neutral pH and four secondary amines that could be partially positively charged in neutral pH and fully charged in acidic pH. We used this cationic lipid for preparing the cationic liposome with the helper lipid 1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine (DOPE) that was shown to improve the lipoplex efficiency [1] and appears to be a favorable helper lipid for DNA transfection *in vitro* [2], [3].

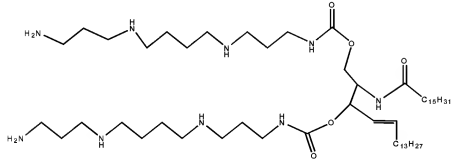


Figure 1: Chemical structure of N-palmitoyl-D-erythro sphingosyl-1-3-spermine (PCDCS).

We used this PCDCS/DOPE formulation both for plasmid DNA and siRNA *in vitro* transfection to different types of the cells. PCDCS/DOPE 1/3 formulation was prepared by mixing 50µl of 10mM PCDCS in Double Distilled Water (DDW) with 150µl of 10mM

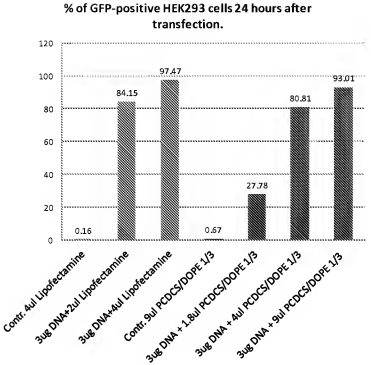
DOPE in Tert-Butanol. The mix was frozen in -80°C and then lyophilized. The hydration of the lipid mix was performed with 500µl 20mM HEPES buffer (pH 7.4) at room temperature. Plasmid DNA was added in 20mM HEPES buffer (pH 7.4). We used a GFP-expressing plasmid for transfection studies. The results were obtained by FACS analysis as a percentage of GFP positive cells 24 hours after transfection. Lipofectamine transfection reagent was taken as a positive control and for comparison as one of the best transfection reagents based on cationic lipid in the market.

First, HEK293 cells were chosen for transfection as the cells with one of the highest transfection efficiency. PCDCS/DOPE 1/3 lyophilized mix was hydrated by 500µl 20mM HEPES buffer (pH 7.4) (resulting in 1 mM PCDCS), and incubated 15 minutes at room temperature. Then 3µg of GFP-expressing plasmid were incubated with different amounts of PCDCS/DOPE cationic liposomes for 20 minutes at room temperature and added to the cells for 24 hours. For comparison and GFP positive control, the plasmid DNA was incubated with different amounts of Lipofectamine transfection reagent for 20 minutes at room temperature and also added to the cells. The results (Figure 2A) were obtained by FACS analysis as percent of GFP positive cells. It was shown that PCDCS/DOPE 1/3 liposome has a high

potential as a transfection reagent in HEK293 that could be as good as of Lipofectamine.

We next tested our new transfection reagent in the other cell lines. The above-mentioned transfection procedure was used with HeLa cells, cultured in DMEM with 10% FCS. As could be concluded from the Figure 2B, transfection efficiency of both Lipofectamine and PCDCS/DOPE 1/3 mix was significantly lower than in HEK293. PCDCS/DOPE 1/3 mix shows also less transfection efficiency than Lipofectamine. In HepG2 cells, transfection efficiency of both Lipofectamine and PCDCS/DOPE 1/3 mix was very low (Data not shown).

A


% of GFP-positive HEK293 cells 24 hours after transfection.
120
100
80
60
40
20
0
0.16
84.15
97.47
0.67
27.78
80.81
93.01
Contr. 4ul Lipofectamine
3ug DNA+2ul Lipofectamine
3ug DNA+4ul Lipofectamine
Contr. 9ul PCDCS/DOPE 1/3
3ug DNA + 1.8ul PCDCS/DOPE 1/3
3ug DNA + 4ul PCDCS/DOPE 1/3
3ug DNA + 9ul PCDCS/DOPE 1/3

B

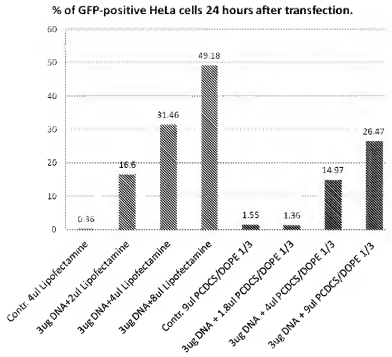


Figure 2: GFP expressing plasmid DNA transfection into HEK293 cells (A) and HeLa cells (B). 3µg of plasmid DNA incubated with 2 and 4 µl Lipofectamine and with 1.8, 4 and 9µl of PCDCS/DOPE liposomes. 4µl Lipofectamine and 9µl of PCDCS/DOPE were used as vehicle control. 24 hours post transfection cells were trypsinized, washed in PBS and analyzed by FACScan. Percent of GFP positive HEK293(A) and HeLa cells (B) was measured.

We decided to test several PCDCS/DOPE molar ratios to find the most optimal for DNA transfection. GFP expressing plasmid DNA was transfected to HeLa cells as described previously with PCDCS/DOPE with several molar ratios (See figure 3).

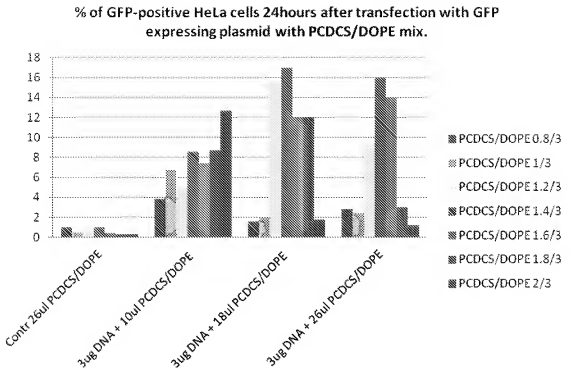


Figure 3: GFP expressing plasmid DNA transfection into HeLa cells. PCDCS/DOPE mixes with different molar ratios (0.8/3, 1/3, 1.2/3, 1.4/3, 1.6/3, 1.8/3 and 2/3) were used. 3µg of plasmid DNA incubated with 10, 18 and 26µl of PCDCS/DOPE liposomes. 26µl of PCDCS/DOPE were used as vehicle control. 24 hours post transfection cells were trypsinized, washed in PBS and analyzed by FACScan. Percent of GFP positive HeLa cells was measured.

The PCDCS/DOPE 1.4/3 molar ratio (Figure 3) appeared to be the most optimal for DNA transfection.

We compared PCDCS as a transfection reagent candidate with another cationic lipid: N-palmitoyl-D-erythro-sphingosyl-1-carbamoyl spermine, also referred to as palmitoyl ceramide

carbamoyl spermine (PCCS) (Figure 4a). It can be seen that PCCS differs from PCDCS in the fact that the latter has only a single polyalkylamine chain, i.e., PCCS possess only one spermine residue as compared with PCDCS, which possess two spermine residues (Figure 1).

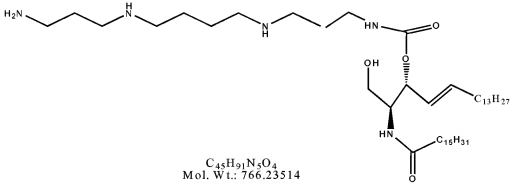


Figure 4: Chemical structure of Palmitoyl ceramide carbamoyl spermine (PCCS). Liposomes were prepared as previously by hydration with 20mM HEPES pH 7.4.

In the next experiment, we compared PCDCS/DOPE 1.4/3 mix to PCCS/DOPE 2/1 mix which was found previously as an optimal molar ratio for DNA transfection for PCCS (Previous data). Cationoic liposomes were prepared with both mixes as previous by hydration of lyophilized mix with 500ml 20mM HEPES pH 7.4. Transfection was performed as described previously and GFP positive HeLa cells were counted and analysed on FACScan (Figure 5). We can conclude from these results that PCDCS

cationic lipid is much more effective for DNA transfection than PCCS.

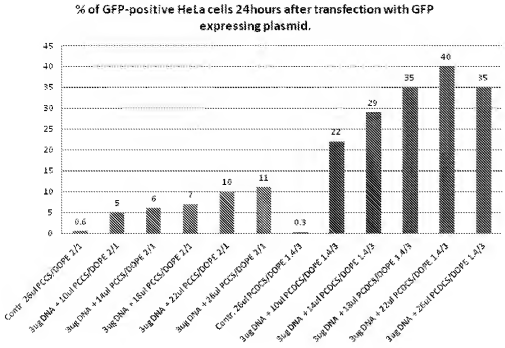


Figure 5: GFP expressing plasmid DNA transfection into HeLa cells. PCDCS/DOPE 1.4/3 and PCCS/DOPE 2/1 lyophilized mixes were hydrated with 500µl 20mM HEPES buffer (pH 7.4) and incubated 15 minutes in room temperature. 3µg of plasmid DNA incubated with 10, 14, 18, 22 and 26µl of PCDCS/DOPE 1.4/3 and PCCS/DOPE 2/1 liposomes, 26µl of PCDCS/DOPE 1.4/3 and PCCS/DOPE 2/1 were used as vehicle control. 24 hours post transfection cells were trypsinized, washed in PBS and analyzed by FACScan. Percent of GFP positive HeLa cells was measured

PCDCS/DOPE mix was also tested as a transfection reagent for siRNA transfection. C26 mouse colon carcinoma cells stably expressing GFP protein (C26-GFP) were chosen for

transfection efficiency testing. Due to relatively long half life time of GFP prtein, the knockdown effect could be observed only 72 hours after siRNA transfection to the cells (our observation, data not shown). In this experiment we also tested the optimal molar ratio of the PCDCS to DOPE for siRNA transfection as it was done for DNA transfection. We performed transfection of anti GFP siRNA to C26-GFP cells as follows: PCDCS/DOPE lyophilized mixes with different lipid molar ratios (PCDCS/DOPE 0.8/3, 1.3, 1.2/3, 1.4/3 and 1.6/3) were hydrated with 500µl 20mM HEPES buffer (pH 7.4) at room temperature. Different amounts of PCDCS/DOPE liposomes were mixed with 200 nmole GFP siRNA in OPTI MEM and added to the cultured C26-GFP cells. After 72 hours, cell were harvested and analyzed by FACScan (Figure 6).

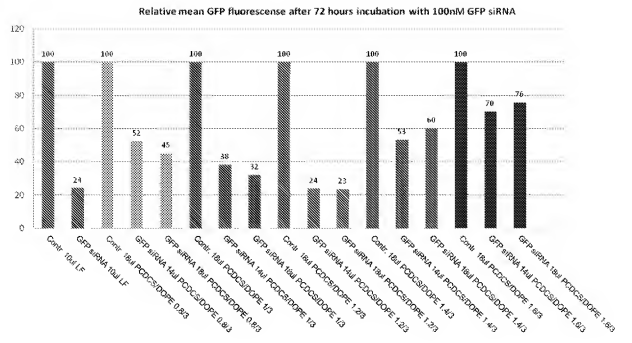


Figure 6: FACScan analysis of GFP expressing C26 cells 72 hours after transfection with anti GFP siRNA. 200 nmole of anti GFP siRNA were mixed with 14 and 18µl of PCDCS/DOPE mixes with different lipid molar ratios: Yellow (columns 3-5): PCDCS/DOPE 0.8/3, Red (columns 6-8): PCDCS/DOPE 1/3, Green (columns 9-11): PCDCS/DOPE 1.2/3, Blue (columns 12-14): PCDCS/DOPE 1.4/3 and Violet (columns 15-17): PCDCS/DOPE 1.6/3; incubated for 30 min and added to the cells. Lipofectamine was used as a positive control. 72 hours after transfection, cells were harvested and analyzed by FACScan. The reduction of mean fluorescense level of GFP positive cells was calculated as % from the control vehicle without siRNA, presented as 100%.

As can be concluded from Figure 7, the optimal molar ratio of PCDCS and DOPE in the mix for siRNA transfection is 1.2 to 3 and it is different from that for DNA transfection (Figure 3). We can conclude also that PCDCS/DOPE mix performed as transfection reagent exactly as Lipofectamine, resulting in

almost 80% knock down activity of transfected siRNA, that characterizes it as a strong transfection reagent for siRNA.

PCDCS/DOPE mix was also compared to PCCS/DOPE mix in siRNA transfection. Transfection of anti-GFP siRNA was performed as in the previous experiment with PCDCS/DOPE 1.2/3 mix and with PCCS/DOPE 2:1 mix. The results are presented on Figure 7.

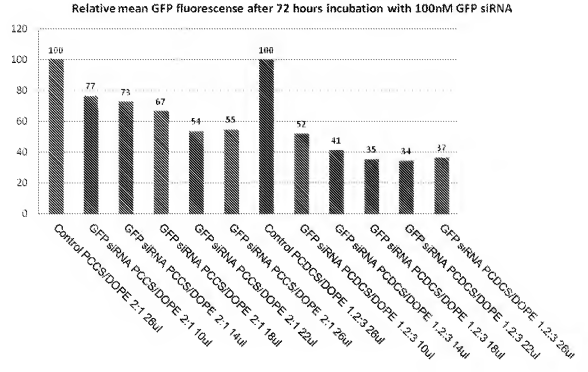


Figure 7: FACScan analysis of GFP expressing C26 cells 72 hours after transfection with anti GFP siRNA. 200 nmole of anti GFP siRNA were mixed with 10, 14, 18, 22 and 26µl of PCDCS/DOPE 1.2/43 mix and PCCS/DOPE 2:1 mix, incubated for 30 min and added to the cells. 72 hours after transfection, cells were harvested and analyzed by FACScan. The reduction of mean fluorescense level of GFP positive cells was calculated as % from the control vehicle without siRNA, presented as 100%.

These results show that as in the case of DNA transfection, despite the lower presence in the mix with DOPE, PCDCS performs as a more efficient transfection reagent for siRNA than PCCS. Taking together, the above results show that PCDCS cationic lipid mixed with DOPE helper lipid appears to be a potential and strong transfection reagent for nucleic acids *in vitro*. As a molecule having two spermine residues in its hydrophilic head, it is more efficient than previously designed PCCS, bearing only one spermine.

The references referred to above are as follows. On information and belief, copies are being submitted on even date herewith:

1. Kerner, M., et al., *Interplay in lipoplexes between type of pDNA promoter and lipid composition determines transfection efficiency of human growth hormone in NIH3T3 cells in culture*. Biochim Biophys Acta, 2001. **1532**(1-2): p. 128-36.

2. Zuidam, N.J. and Y. Barenholz, *Electrostatic parameters of cationic liposomes commonly used for gene delivery as determined by 4-heptadecyl-7-hydroxycoumarin*. Biochim Biophys Acta, 1997. **1329**(2): p. 211-22.

3. Zuidam, N.J. and Y. Barenholz, *Electrostatic and structural properties of complexes involving plasmid DNA and cationic lipids commonly used for gene delivery*. Biochim Biophys Acta, 1998. **1368**(1): p. 115-28.

The undersigned hereby further declares that all statements made herein of my own knowledge are true and that all

statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

_____11/12/2011_____	_____/Kirill Makedonski/___
Date	Kirill Makedonski